ESC European Society of Cardiology

Cardiovascular Research (2021) **117**, 2326–2339
doi:10.1093/cvr/cvab046

REVIEW

Vascular smooth muscle cells in atherosclerosis: time for a re-assessment

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Received 20 August 2020; editorial decision 11 January 2021; accepted 4 February 2021; online publish-ahead-of-print 8 February 2021

Abstract

Vascular smooth muscle cells (VSMCs) are key participants in both early and late-stage atherosclerosis. VSMCs invade the early atherosclerotic lesion from the media, expanding lesions, but also forming a protective fibrous cap rich in extracellular matrix to cover the 'necrotic' core. Hence, VSMCs have been viewed as plaque-stabilizing, and decreased VSMC plaque content—often measured by expression of contractile markers—associated with increased plaque vulnerability. However, the emergence of lineage-tracing and transcriptomic studies has demonstrated that VSMCs comprise a much larger proportion of atherosclerotic plaques than originally thought, demonstrate multiple different phenotypes *in vivo*, and have roles that might be detrimental. VSMCs down-regulate contractile markers during atherosclerosis whilst adopting alternative phenotypes, including macrophage-like, foam cell-like, osteochondrogenic-like, myofibroblast-like, and mesenchymal stem cell-like. VSMC phenotypic switching can be studied in tissue culture, but also now in the media, fibrous cap and deep-core region, and markedly affects plaque formation and markers of stability. In this review, we describe the different VSMC plaque phenotypes and their presumed cellular and paracrine functions, the regulatory mechanisms that control VSMC plasticity, and their impact on atherogenesis and plaque stability.

Keywords Atherosclerosis • Vascular smooth muscle

1. Introduction

Atherosclerosis is a chronic inflammatory disease characterized by formation of lipid-laden plaques in the arterial wall. Plaques can narrow the blood vessel (stenosis) or obstruct blood flow by thrombi forming upon plaque rupture or erosion, leading to myocardial infarction.[1] Despite major therapeutic advances and primary prevention, atherosclerotic cardiovascular disease remains the leading cause of death worldwide.[2] Besides their lipid content, atherosclerotic plaques comprise different cell types including vascular smooth muscle cells (VSMCs) and inflammatory cells (e.g. macrophages, dendritic cells, and lymphocytes), extracellular matrix (ECM) proteins, and cellular debris that accumulates in the deep cholesterol-rich 'necrotic' core.[1]

An unstable and rupture-prone plaque is defined by a collagen-poor thin fibrous cap infiltrated by macrophages and lymphocytes with rare or no VSMCs and a relatively large underlying necrotic core.[1] This definition implies that macrophages are over-represented in unstable plaques relative to VSMCs and are considered detrimental for plaque stability, whereas VSMCs are regarded as plaque-stabilizing. However, more recent studies using rigorous lineage-tracing systems have indicated that this concept may be an oversimplification, and that VSMCs comprise a much greater proportion of plaque cells than previously thought. In particular, VSMCs lose their own lineage markers and adopt macrophage and other markers, making studies based entirely on identification of static markers unreliable. In contrast to ruptured plaques, lesions with superficial plaque erosion contain few macrophages but abundant VSMCs and ECM (e.g. hyaluronan) without disruption of the fibrous cap.[3] VSMCs from eroded plaques show reduced expression of VSMC differentiation markers[4] yet their true role and phenotype during plaque erosion is still unclear and requires further investigation. Following recent new insights in the dynamic phenotype of VSMCs in atherosclerosis, we believe it is time for a re-assessment of the complex role of VSMCs in atherogenesis and plaque stability. In particular, VSMCs are able to populate both the fibrous cap and plaque core, and have different effects

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on atherogenesis by adopting different cellular phenotypes. In this review, we discuss the distinct phenotypes that VSMCs acquire in the atherosclerotic milieu, the mechanisms that govern these phenotypic switches, their secretory and paracrine functions, and their pathological impact on atherosclerosis.

2. Identification of plaque VSMCs

VSMCs are the major component of the vessel wall responsible for maintaining vascular tone and regulating blood pressure via their contractile properties.[5] The ultrastructural characteristics of medial VSMCs have been carefully studied in the early 1970s, revealing remarkable cell plasticity, which still remains valid today. In normal healthy arteries, VSMCs have a spindle-like shape, are densely packed with microfilaments and express a range of proteins [e.g. smooth muscle alpha actin 2 (ACTA2), transgelin (TAGLN), and myosin heavy chain 11 (MYH11)] required for their contractile function.[5–7] Upon vascular injury[8] or atherosclerosis development,[9] contractile marker gene expression is downregulated in VSMCs in part through activation of a G/C repressor element in contractile gene promoters.[10] Until recently, identification of VSMCs within lesions largely relied on the detection of contractile markers, usually by means of immunohistochemistry, meaning that cells of VSMC origin that lack these markers were not identified.

Recent advances in genetically engineered mouse models have allowed us to label and track VSMCs with more fidelity in different disease models including atherosclerosis, even when VSMC characteristics and contractile protein markers are lost. A common example comprises a mouse containing a tracking marker (usually a fluorescent reporter transgene), crossed with a mouse containing the promoter of a VSMC-specific contractile gene (e.g. Myh11) linked to a drug-inducible (e.g. tamoxifen) recombinase system. Drug-induced recombination permanently marks all VSMCs and their progeny, allowing tracking of cells of VSMC origin even when MYH11 expression is lost. Studies using these models have shown that >80% of VSMC-derived cells in mouse plaques are negative for ACTA2,[11] demonstrating that immunohistochemistry for ACTA2 alone is not adequate to quantify mouse plaque VSMCs. Similarly, the majority of plaque cells in human coronary lesions identified as being of VSMC origin based on a VSMC-specific epigenetic signature fail to express ACTA2 and MYH11.[12] Moreover, although most ACTA2^{+} cells can be found in the fibrous cap, VSMC-derived ACTA2^{-} cells accumulate in the plaque core,[13] disputing the view that the core is predominantly occupied by macrophages. Not only have we underestimated the VSMC content of plaques, we likely have also misidentified VSMCs as macrophages. For example, both *in vitro* experiments and (electron) microscopic analysis of human plaques have shown that VSMCs can transform into foam cells upon cholesterol loading,[14–16] thereby expressing the scavenger receptor CD68,[16] a widely used marker to identify macrophages, and multiple studies reveal that ~40% of CD68^{+} cells in human and mouse plaques are of VSMC and not myeloid origin.[11,16] Indeed, VSMCs have been shown to adopt a macrophage-like phenotype and express multiple markers of macrophages, such as CD11b and galectin-3 (LGALS3).[16–18] The major advantage of these lineage-tracing models is that they allow us to study both VSMC origin and fate, including the ability to switch to different phenotypes (*Figure 1*), thereby re-assessing and improving our understanding of their role in atherosclerosis (*Figure 2*).

3. Origin of plaque VSMCs

3.1 Embryonic origin

Lineage-tracing studies have unambiguously demonstrated that VSMCs in the arterial wall originate from multiple progenitors from different embryonic origin, creating a mosaic with a segmental architecture of the thoracic artery.[19,20] VSMCs from the ascending aorta, aortic arch and carotid arteries derive from the neural crest, while the descending aorta predominantly originates from somitic precursors. The secondary heart field contributes to the development of the proximal aortic sinus overlapping neural crest-derived VSMCs, but the coronary arteries are formed by the epicardium.[20]

3.2 Local media-derived VSMCs

Wisler proposed in 1968 that VSMCs invade the arterial intima in humans and experimental animals by migration and proliferation from the arterial media.[21] Although human arteries also contain intimal VSMCs, the conventional view has been that VSMCs in human atherosclerotic plaques also derive from local medial VSMCs that dedifferentiate when invading the intima.[22] VSMC migration into the intima is hard (but not impossible) to measure directly, and a number of kinetic studies identified a pool of migrating VSMCs, some of which do not proliferate in rodent models of neointima formation.[23] However, the introduction of rigorous lineage-tracing models have provided solid evidence that VSMCs undergo phenotypic switching during atherosclerosis, originate almost exclusively from the local arterial wall, and comprise up to 30–70% of all plaque cells.[11,13,17] These studies also refuted previous ideas that VSMCs originate predominantly from the bone marrow, and all circulating cell types have now been excluded as primary sources of plaque VSMCs.[24] Although bone marrow-derived cells can still express ACTA2 and other VSMC markers both *in vitro* and *in vivo*,[18] they may not behave like vessel wall-derived VSMCs.[25]

A long-standing and important concept in atherosclerosis has been that VSMCs are derived from a small patch or clone that expands in the intima, such that plaque VSMCs are monoclonal or oligoclonal. VSMC clonality has been demonstrated in human atherosclerotic plaques using biochemical and molecular analyses.[26] More recently, VSMC lineage studies using multicolour reporters have confirmed that mouse plaque VSMCs are also oligoclonal,[13,19,27] indicating that from a mixed population of medial VSMCs only a few VSMC clones give rise to the whole VSMC population in the plaque. These few VSMCs populate the fibrous cap, but are highly proliferative and also invade the plaque core.[19] The mechanisms that drive clonal VSMC expansion are under study, and include a number of possibilities, including: (i) cell-autonomous properties, where the cell is 'primed' or 'selected' to invade the lesion, (ii) a stochastic event, where the cell is located at the 'right time, right place' to break through the fragmented elastic lamina, (iii) competition, where certain clones prevent other VSMCs participating, (iv) selective disadvantage due to VSMC senescence (see Section 5.1.3), apoptosis (see Section 5.3.1), or efferocytosis, and (v) presence of a 'progenitor' cell type in the media ready to respond.

3.3 Mesenchymal stem cell and endothelial cell-derived VSMCs

Although it is now generally accepted that local medial VSMCs provide the major contribution of VSMCs to neointima formation upon vessel injury,[8] several studies suggest that other sources within the vessel wall also contribute, including adventitial mesenchymal stem cells (MSCs),[28]

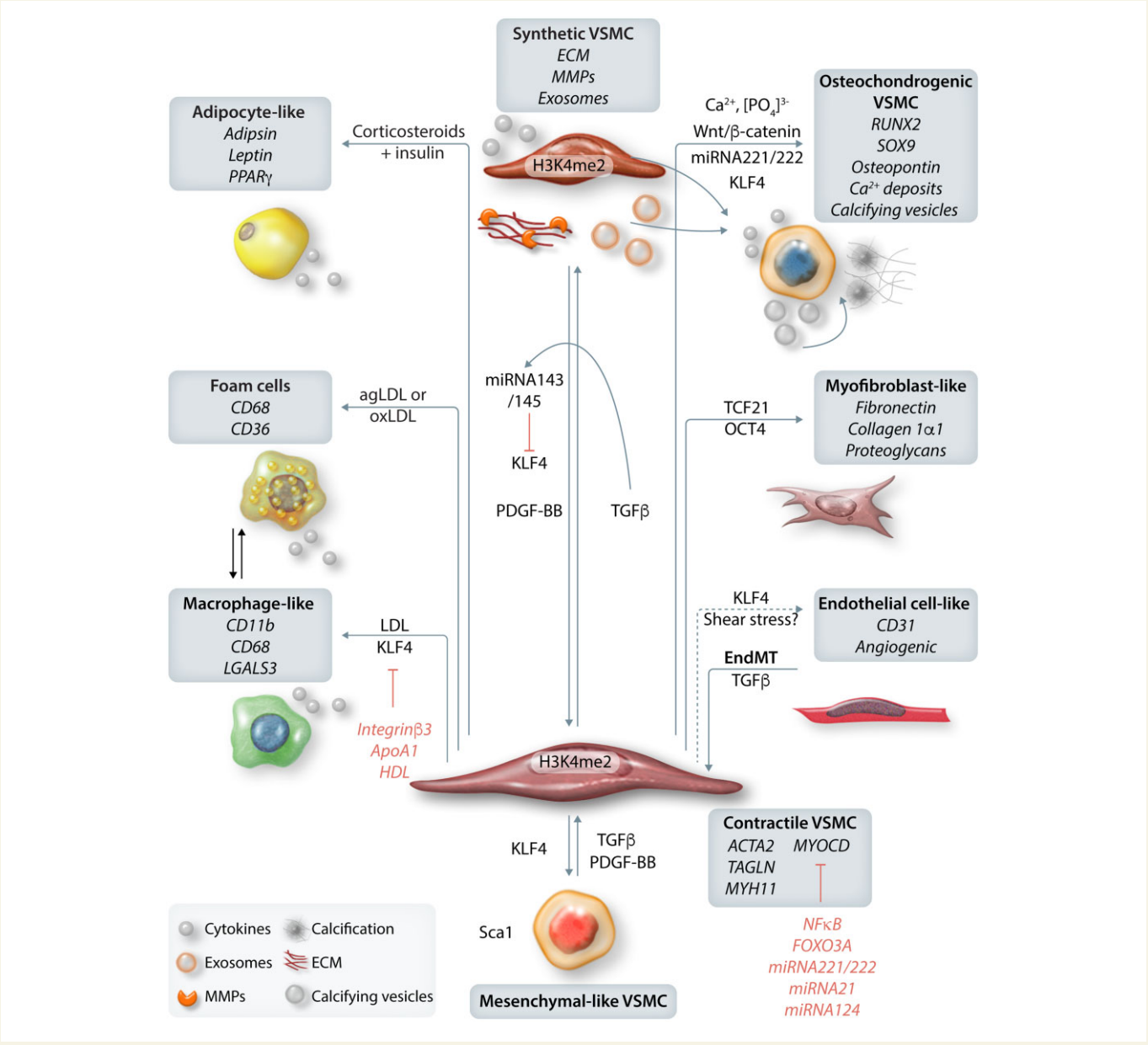

Figure 1 Regulation and characterisation of VSMC phenotypic switching. In the healthy vessel wall, VSMCs exhibit a contractile phenotype characterized by expression of contractile proteins (ACTA2, TAGLN, and MYH11) regulated by MYOCD, which is negatively regulated by NFκB, FOXO3A, miRNA221/222, miRNA21, and miRNA124. Upon injury or atherosclerosis, VSMCs switch to a synthetic phenotype mediated via PDGF-BB and Klf4, which is inhibited by miRNA143/145 and TGFβ. Synthetic VSMCs are characterised by increased secretion of ECM, MMPs, pro-inflammatory cytokines, and exosomes. Exosomes can trigger neighbouring VSMCs to transdifferentiate into osteochondrogenic VSMCs characterised by RUNX2, SOX9, osteopontin expression, release of calcium deposits, and calcifying vesicles. Osteochondrocyte-like VSMCs secrete calcifying vesicles that further propagate calcification. In some cases, calcifying VSMCs originate from medial contractile VSMCs in response to high concentrations of calcium and phosphate, β-catenin/Wnt signalling, KLF4, or miRNA221/222. Exposure to agLDL or oxLDL triggers a switch to macrophage-like VSMCs or foam cells characterised by CD68, CD36, LGALS3, CD11b, and pro-inflammatory cytokine expression, which is inhibited by integrin β3, apolipoprotein A1, or HDL. VSMCs may adopt adipocyte-like features in a corticosteroid/insulin-rich milieu characterised by expression of adipsin, leptin, and PPARγ pathway activation. KLF4 promotes phenotypic modulation into macrophage-like and Sca1-expressing mesenchymal-like VSMCs, while MSCs differentiate into VSMCs *in vitro* when exposed to TGFβ and PDGF-BB. VSMCs might also derive from ECs following EndMT driven by TGFβ signalling. VSMCs can potentially transdifferentiate into endothelial-like cells characterised by CD31 expression and angiogenic features in response to shear stress or via a KLF4-dependent stem cell state. TCF21 and OCT4 promote phenotypic modulation into an athero-protective 'myofibroblast'-like VSMC characterised by fibronectin, collagen 1 alpha 1, and proteoglycans production. Phenotypically switched VSMCs conserve an epigenetic H3K4me2 signature of their former contractile state that can be used as a VSMC-specific marker. MYOCD, myocardin; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; FOXO3a, forkhead box O3a; PDGF-BB, platelet-derived growth factor; KLF4, Krüppel-like factor 4; ECM, extracellular matrix; MMP, matrix metalloproteinases; RUNX2, runt-related transcription factor 2; SOX9, SRY-related HMG-box 9; PPARγ, peroxisome proliferator-activated receptors gamma; Sca1, stem cell antigen 1; TGFβ, transforming growth factor beta; MSCs, mesenchymal stem cells; EndMT, endothelial-to-mesenchymal transition; TCF21, transcription factor 21; OCT4, octamer binding transcription factor 4.

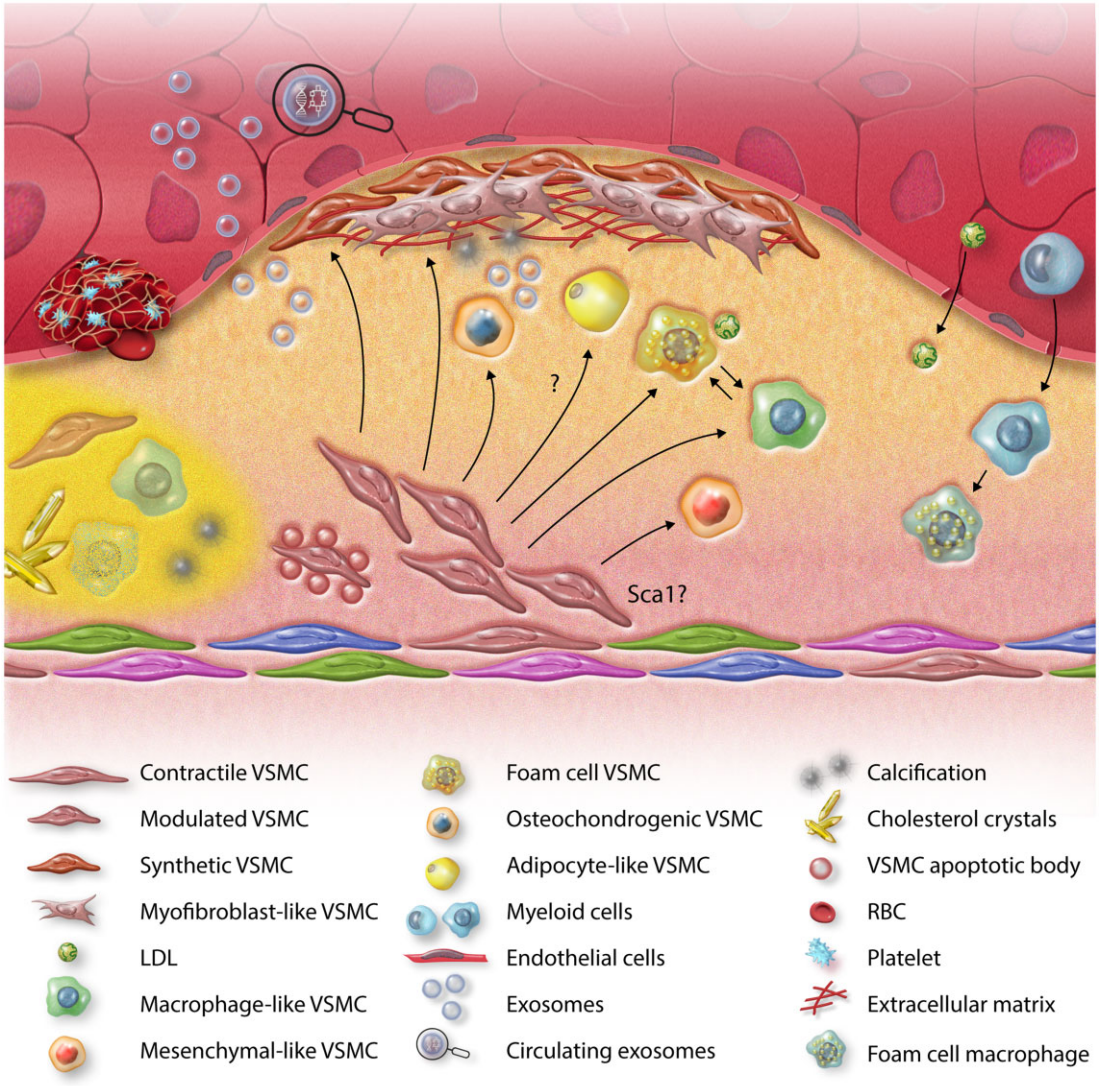

Figure 2 The role of VSMCs in the pathogenesis of atherosclerosis. Schematic of all VSMCs phenotypes found in mouse and/or human atherosclerotic plaques to date including their location within the lesion, although their presence and/or abundance may vary depending on the lesion stage. The medial layer is composed of different VSMC clones (illustrated as red, blue, purple, and green) exhibiting a contractile phenotype. During atherogenesis, medial VSMCs underlying the lesion lose their contractile markers and undergo phenotypic switching, likely upon exposure to lipids or calcification-promoting molecules. Only one or a small number of phenotypically modulated medial VSMC clones (here in red) invade and occupy the lesion. The mechanism for oligoclonal expansion is still unclear but may involve a subpopulation of Sca1^{+} VSMCs that are predisposed to invade the lesion or express Sca1 during clonal expansion. Within the lesion, these modulated VSMCs adopt various phenotypes: synthetic, macrophage-like, foam cells, MSC-like, osteochondrogenic, myofibroblast-like/fibromyocyte and potentially adipocyte-like, and EC-like VSMCs. Synthetic and myofibroblast-like/fibromyocyte VSMC clones occupy the fibrous cap and produce ECM. Macrophage-like and foam cell-like VSMCs possess requisite markers but still differ from professional monocytes that enter the lesion via the circulation. In response to various cues, VSMC undergoes apoptosis characterised by formation of apoptotic bodies and/or undergo (secondary) necrosis and accumulate in the cholesterol-rich 'necrotic' core. Osteochondrogenic VSMCs promote micro- and macro-calcification that accumulate in the fibrous cap and necrotic core, respectively. Calcifying and synthetic VSMCs secrete EVs or exosomes carrying various cargos (e.g. nucleic acids and bioactive molecules) that are released into the circulation upon plaque rupture and can potentially be used as CVD biomarkers. Sca1, stem cell antigen 1; ECM, extracellular matrix.

pericytes[29] and fibroblasts,[30] or endothelial cells (ECs).[31,32] For example, adventitial cells expressing MSC markers, such as stem cell activating antigen 1 (Sca1) can differentiate into VSMCs expressing SMC promoter-driven reporter genes and contractile markers *in vitro* and in vein grafts,[28] Glioma associated oncogene 1^{+} adventitial MSCs generate ACTA2^{+} cells that migrate and proliferate in atherosclerotic plaques in ApoE$^{-/-}$mice,[33] the adventitia and media of ApoE$^{-/-}$mice contain a subpopulation of cells expressing the coronary artery disease (CAD)-associated transcription factor 21 (TCF21) and give rise to ACTA2^{+} and TAGLN^{+} cells in the fibrous cap,[34] and a subset of medial MYH11^{+} VSMCs expressing Sca1 undergo phenotypic switching in mouse plaques.[35] The Sca1^{+} VSMC population increases upon vascular injury and their transcriptional

profile is similar to the synthetic plaque Sca1$^+$ VSMC lineage. These Sca1$^+$ medial VSMCs may be predisposed to proliferate and give rise to phenotypically distinct cells within plaques (*Figure 2*), but whether this Sca1$^+$ population truly controls clonal VSMC expansion remains to be determined. There is also accumulating evidence that some plaque VSMCs derive from ECs following endothelial-to-mesenchymal transition (EndMT). EndMT, driven by transforming growth factor beta (TGFβ) signalling, oscillatory shear stress, oxidative stress, and hypoxia, promotes progression of experimental atherosclerosis and correlates with an unstable plaque phenotype in humans.[31,32] Although these studies provide in-depth insights into VSMC diversity, additional research is needed to further explore VSMC heterogeneity in relation to their function in vascular disease.

3.4 Source of VSMCs in human atherosclerosis

The source and fate of VSMCs in human atherosclerosis is more difficult to determine, as lineage- tracing studies are harder to perform. However, identification of cells of VSMC origin in human plaques is possible by detecting an epigenetic marker specific to VSMCs (H3K4me2 of the Myh11 locus) on histological sections and persists upon phenotypic switching.[12] Phenotypically modified VSMCs have also been identified in single-cell RNA-seq (scRNA-seq) datasets from human coronary[36,37] and carotid[38,39] artery lesions and the transcriptomic cell clusters of advanced human coronary lesions are remarkably similar to those of carotid plaques despite their differential developmental origin[38] and to those of mouse brachiocephalic artery plaques.[37]

However, there are many differences between the human and mouse arterial intima. For example, the normal human intima contains pre-existing VSMCs and diffuse intimal thickenings develop early in life before atherosclerosis development.[40] Compared to medial VSMCs, these pre-existing intimal VSMCs have different transcriptomic profiles[41] and display a more synthetic phenotype when exposed to various stress signals.[26,42] The contribution of VSMCs from pre-existing intimal VSMCs vs. underlying medial VSMCs to human atherosclerotic plaques is not known, and whether they also undergo clonal expansion is still unclear. Overall, the complex differences between medial, intimal and plaque VSMCs, including the distinct localisation of atherosclerotic lesions and positional identity of VSMCs in humans vs. mice, could influence VSMC plasticity and their ultimate role in atherosclerosis.

4. Phenotype of plaque VSMCs

4.1 VSMC phenotypic switching

The term phenotypic switching is often used to describe de-differentiation of a contractile VSMC to a synthetic state, but can refer to any type of phenotypic change to an alternative non-contractile VSMC phenotype. Although VSMCs within atherosclerotic plaques are oligoclonal, each VSMC clone appears to have a high level of plasticity, and is capable of transforming into different phenotypes (*Figure 1*).[11,13,27,35] While these phenotypes have been known for many years, scRNA-seq has allowed clusters of VSMCs to be identified expressing a transcriptional profile that corresponds to these phenotypes. Phenotypic switching is generally thought to occur in the intima; however, medial VSMCs underlying lesions also lose contractile markers in atherosclerosis[9] and after vascular injury,[8] likely in response to lipids or mitogens released after injury.[17] Medial VSMC phenotypic switching may occur before migration and proliferation, and independently of clonal expansion.[26,27,36] VSMCs can adopt a wide range of phenotypes in the intima and media, and VSMCs can alternate between different phenotypes in response to various environmental cues, with different effects on atherogenesis (*Figure 2*) and markers of plaque stability (*Figure 3*).[26,43] Importantly, phenotypic switching seems to be a (at least partly) reversible process and thus therapeutically modifiable.[38]

4.1.1 Contractile vs. synthetic VSMCs

The switch from contractile to synthetic phenotype is defined by loss of contractile markers (e.g. ACTA2, MYH11, TAGLN and calponin) with gain of synthetic organelles, migratory and proliferative properties, and plays a critical role in both neointimal hyperplasia and atherogenesis[42] (*Figure 3*).[26] Historically, this phenotypic switch was viewed as the hallmark of vascular repair, a reversible process wherein VSMCs return to their contractile state after completing vessel repair.[5,44] For example, neointimal VSMCs re-acquire their contractile markers after injury in a rat balloon injury model.[45] In contrast, VSMCs retain their non-contractile status during atherosclerosis due to the continuous exposure to phenotypic switching-inducing stimuli, although they may maintain their ability to revert back to their contractile phenotype.[38]

4.1.2 Foam cells

Historically, foam cells were viewed as bone marrow-derived macrophages in fatty deposits in artery walls, where they ingest low-density lipoproteins (LDL), giving them a foamy appearance. While macrophages do contribute to foam cells within atherosclerotic plaques, VSMCs also transform into foam cells when exposed to aggregated or oxidised LDL in the intima and media of hyperlipidaemic mice and humans,[16,46] and the majority of foam cells in atherosclerotic plaques appear derived from VSMCs.[16,46] Lipid uptake traditionally occurs through specialized scavenger receptors (e.g. SR-A and CD36), but may also involve alternative pathways, such as phagocytosis and autophagy.[27,47] While clearance of lipid species may be beneficial at first, lipid loaded-VSMCs in culture secrete a variety of pro-inflammatory mediators[48] and undergo apoptosis from free cholesterol overload,[49] likely compromising plaque stability (*Figure 3*). The cholesterol excess in VSMCs is likely the result of inadequate cholesterol efflux due to reduced expression of the ABCA1 efflux transporter[16,46] and/or impaired autophagy in advanced plaques.[50]

4.1.3 Macrophage-like

Macrophage-like VSMCs occur in both human[11,16] and mouse[17,27] atherosclerotic plaques, and also in the media of atherosclerotic mice.[26] The switch to a macrophage-like cell is likely driven by lipid accumulation, as cholesterol loading transdifferentiates VSMCs into macrophage-like cells in culture,[51] and can be reversed by stimulating cholesterol efflux via apolipoprotein A1 and high-density lipoprotein (HDL).[17] Macrophage-like VSMCs up-regulate 'macrophage markers', such as LGALS3, CD68,[51] and pro-inflammatory cytokines [e.g. monocyte chemoattractant protein 1 (MCP1)],[11] yet not all lipid-laden VSMCs adopt a macrophage-like phenotype *in vivo*[36] and not all plaque VSMCs expressing LGALS3 adopt a macrophage-like state.[37] Instead, LGALS3 is expressed in >60% of mouse plaque VSMCs during a transition state to a non-macrophage but pro-inflammatory and osteochondrogenic (see Section 4.1.5) VSMC phenotype, and may be the first cell type to invest the plaque.[37] Hence, the question arises whether macrophage-like VSMCs are as harmful for plaque stability as their myeloid counterparts. Indeed, macrophage-like VSMCs are transcriptionally and functionally different from myeloid-derived macrophages in culture[17,52] and emerge

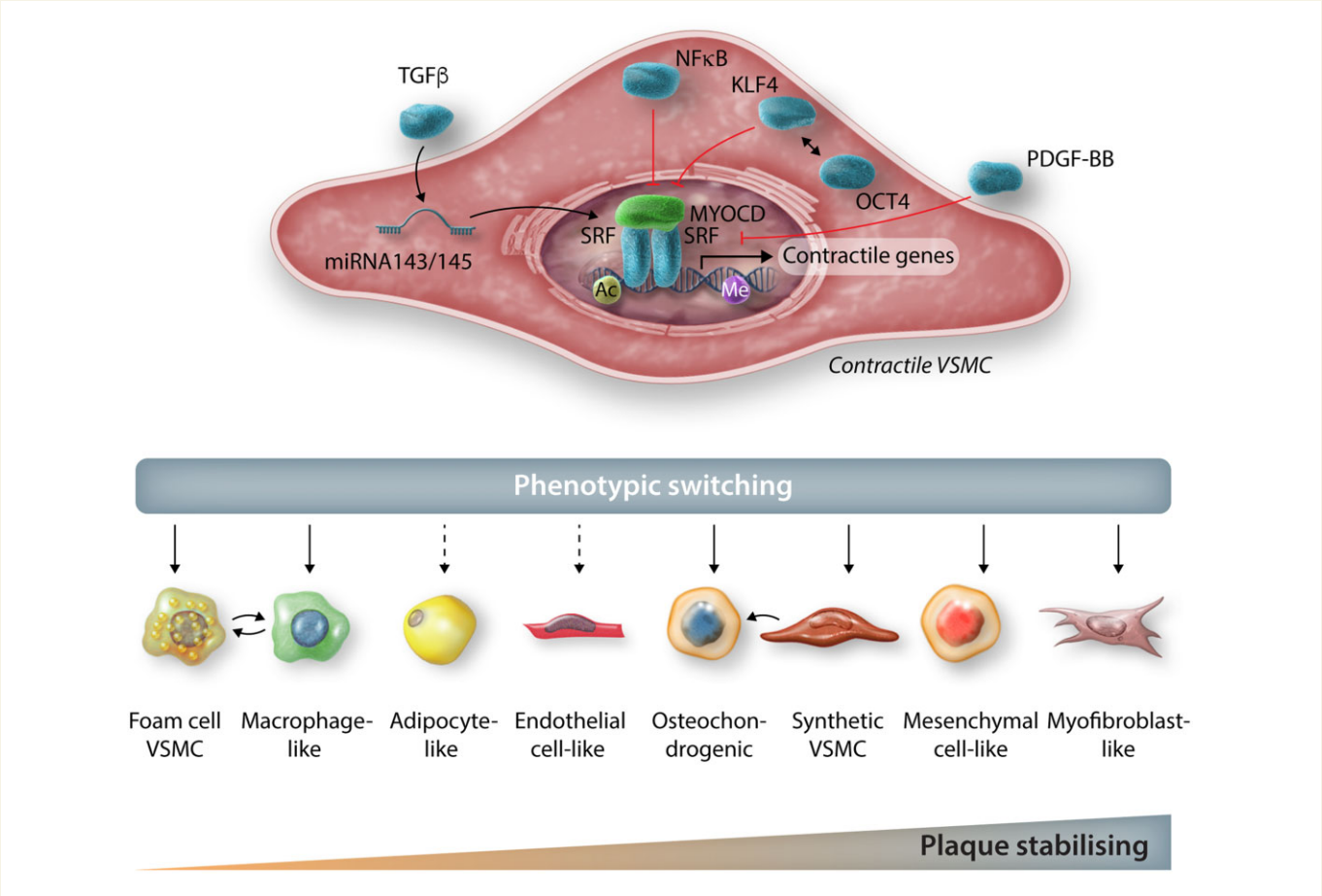

Figure 3 Regulatory pathways and putative role of different VSMC phenotypes on plaque stability. The major regulatory pathways controlling the transition from contractile to phenotypically modified VSMCs include the pluripotency factors KLF4 and OCT4, growth factors PDGF-BB and TGFβ, and NFκB. KLF4 represses expression of contractile genes via multiple mechanisms including binding to SRF, thereby antagonizing MYOCD tethering to SRF. OCT4 is transcriptionally regulated by KLF4 but plays an opposite role in VSMC phenotypic switching. NFκB decreases MYOCD expression by binding to its promoter leading to reduced expression of contractile genes. PDGF-BB suppresses contractile gene expression via different mechanisms, which partially involves KLF4. The contractile phenotype is positively controlled by the growth factor TGFβ and miRNAs miR143 and miR145 that work partially in concert to down-regulate klf4. Also epigenetic regulation by histone acetylation (Ac) and methylation (Me) at the promoter of contractile genes facilitates VSMC differentiation by increasing chromatin accessibility for MYOCD/SRF complexes. Phenotypic switching results in a range of different VSMC phenotypes, some of which are postulated to have predominantly negative effects (foam cell and macrophage-like), neutral effects (osteochondrogenic or synthetic VSMCs), or mostly positive effects on plaque stability (mesenchymal cell- or myofibroblast-like/fibromyocyte cells). Solid evidence for EC-like and adipocyte-like VSMCs in atherosclerosis is currently lacking. To date, only myofibroblast-like VSMCs have been truly associated with plaque stability in mice, although the ability of VSMCs to undergo reprogramming into a stem cell-like state has potential to transdifferentiate into athero-protective VSMC subtypes. TGFβ, transforming growth factor beta; PDGF-BB, platelet-derived growth factor; KLF4, Krüppel-like factor 4; OCT4, octamer binding transcription factor; SRF, serum response factor; MYOCD, myocardin.

later in atherogenesis than myeloid-derived macrophages that enter via the circulation.[38] Overall, the presence of macrophage markers in VSMCs does not imply full functionality as macrophages nor cell specificity, making it difficult to predict their impact on atherosclerotic plaque growth and stability. Nevertheless, based on their pro-inflammatory profile, macrophage-like VSMCs are likely detrimental for plaque stability (*Figure 3*), although their reversibility might offer the opportunity to therapeutically inhibit phenotypic switching.

4.1.4 Adipocyte-like

Lipid accumulation in VSMCs may result from both uptake (and impaired degradation or efflux) of extracellular lipoproteins and *de novo* lipogenesis of fatty acids via peroxisome proliferator-activated receptors gamma and the liver X receptor pathway.[53] For example, human VSMCs cultured in an adipogenic differentiation medium transdifferentiate into adipocyte-like cells expressing adipsin and leptin. Although VSMCs do not fully develop into mature adipocytes, lipid-containing VSMCs express elevated adipsin and lipogenesis genes in human atherosclerotic plaques.[53] Exposure of mouse VSMCs to a similar adipogenic cocktail promotes VSMC proliferation and activation of pro-inflammatory and pro-fibrotic pathways,[54] although whether this is associated with a switch to adipocyte-like VSMCs is unclear. These studies emphasize again the high level of plasticity of VSMCs, but the role and abundance of adipocyte-like VSMCs in atherogenesis is unclear, illustrated by a postulated neutral-to-negative position on the 'plaque-stability scale' (*Figure 3*).

4.1.5 Osteochondrogenic

Vascular calcification is characterised by hydroxyapatite deposits in the vessel wall, and is associated with increased CVD risk.[55] Vascular calcification is a tightly regulated process, primarily driven by VSMCs developing an osteochondrogenic phenotype in response to high levels of calcium and phosphate,[56,57] and lineage-tracing studies in mice show that 98% of all osteochondrogenic cells in plaques are VSMC-derived.[58] Osteochondrogenic VSMCs up-regulate osteochondrogenic markers [e.g. runt-related transcription factor 2 (RUNX2), SRY-related HMG-box (SOX9), and osteopontin)], deposit calcification-prone matrix (e.g. collagen type II and X), and secrete calcifying vesicles.[57] Simultaneously, cells down-regulate expression of molecules that inhibit calcification (e.g. vit K-dependent matrix Gla-protein and fetuin A). However, it is unclear whether contractile VSMCs can directly transform into an osteochondrogenic phenotype[57] or first have to undergo a switch to a synthetic phenotype,[59,60] or an intermediary LGALS3^{+} cell state.[37] Vascular calcification occurs in the intima of atherosclerotic plaques but also in the media where it promotes arterial stiffness.[61,62] Moreover, mouse and human coronary VSMCs with an osteogenic gene signature are associated with plaque destabilisation.[37] In particular, micro-calcified deposits (<50 μm) are associated with increased inflammation and mostly observed in the fibrous cap of human lesions, and thus considered detrimental for plaque stability. In contrast, macrocalcifications (>200 μm) often accumulate in the deep intima or necrotic core in organized structures and may promote plaque stability.[63] Interestingly, cultured calcifying VSMCs are still functionally different from mature, bone-forming osteoblasts, and more susceptible to apoptosis.[64] The opposite roles of micro- and macro-calcification in atherosclerosis limits its therapeutic potential and is illustrated by a neutral position on the 'plaque stability scale' (*Figure 3*).

4.1.6 Myofibroblast-like

A myofibroblast-like or 'fibromyocyte'[36] refers to differentiation of a contractile VSMC into a fibroblast-like cell. Myofibroblast-like VSMCs exhibit a fibroblast-like transcriptional signature characterised by increased expression of fibronectin 1, osteoprotegerin, collagen type 1α1, and various small proteoglycans, including up-regulation of fibroblast-specific pathways. These myofibroblast-like cells have been identified in mouse plaques, particularly accumulating in the fibrous cap, and in a scRNA-seq dataset from human coronary plaques.[36] Differentiation into fibromyocytes is regulated, at least partially, by the CAD-associated transcription factor TCF21. Reduced TCF21 expression is associated with increased CVD risk, and loss of Tcf21 inhibits phenotypic modulation and attenuates fibromyocyte number in the fibrous cap, suggesting an athero-protective role (*Figure 3*).[36] This study also showed that the majority of VSMCs undergo phenotypic modulation into one main cell type rather multiple different phenotypes,[27,37,38] that phenotype modulation can occur in the media, and that genes that are causally associated with CVD can fundamentally alter VSMC phenotype switching *in vivo*.

4.1.7 EC-like

A limited number of studies report that contractile VSMCs can transdifferentiate into an EC-like phenotype. For example, mouse VSMCs cultured under laminar shear stress show increased expression of EC markers (CD31, von Willebrand factor, and VE-cadherin).[65] More recent work shows that human VSMCs can convert into ECs in culture, albeit indirectly, via an intermediary progenitor state elicited by stem cell reprogramming factors,[66] with further differentiation into ECs regulated via Notch signalling. Interestingly, these VSMC-derived ECs exhibit fully fledged EC functions and express CD31 *in vitro* and *in vivo* but could potentially promote intraplaque neovascularisation and haemorrhage associated with human plaque rupture[67](*Figure 3*). Direct evidence of endothelial-like VSMCs in human atherosclerosis is currently lacking, although multipotent VSMC-derived intermediary cells expressing endothelial markers were found in a scRNA-seq dataset from mouse and human plaques.[38]

4.1.8 MSC-like

A rare population of MSC-like Sca1^{+} cells has been identified in medial differentiated VSMCs, which is increased upon vessel injury and exposure to stimuli known to induce phenotypic switching.[35] These medial Sca1+ VSMCs might represent a 'first responder' subpopulation that could undergo or regulate clonal expansion in atherosclerosis in response to environmental cues. Differentiated medial VSMCs can also give rise to adventitial Sca1^{+} progenitor cells that contribute to adventitial remodelling.[68] The VSMCs undergo a 'reprogramming process' controlled by Krüppel-like factor 4 (KLF4) and can transdifferentiate into different cell types, including macrophage-like, EC-like, chondrocyte-like, and adipocyte-like under defined *in vitro* conditions. Although these studies identify VSMC migration in opposite directions, they highlight the ability of Sca1^{+} VSMC-derived cells to promote VSMC transdifferentiation and underline the extremely high degree of plasticity of VSMCs. The highly plastic nature of mesenchymal-like VSMCs offers the opportunity to therapeutically push these cells into a plaque-stabilising phenotype (*Figure 3*).

4.2 Regulation of VSMC phenotype

The list of factors that regulate VSMC de-differentiation, proliferation, and migration is extremely long and covered by some excellent and more extensive reviews.[69–71] We will therefore focus on those known to regulate VSMC phenotypic switching, including TCFs, post-transcriptional regulation by microRNAs, epigenetic modifications, and various environmental stimuli.

4.2.1 Transcriptional

The expression of contractile genes is controlled by the serum response factor (SRF) and its co-activator myocardin (MYOCD), the former binding as a homodimer to the DNA consensus sequence CC(A/T)6GG known as a CArG box within the promoters of contraction-related genes.[72] SRF is ubiquitously expressed and cannot activate transcription of contractile genes alone; binding to MYOCD—which is selectively expressed in VSMCs and cardiac muscle—is required to promote contractile gene transcription.[73] Many factors that control VSMC phenotypic switching alter MYOCD expression or activity either by direct binding to the Myocd promoter or interfering with its binding to SRF (*Figures 1 and 3*). MYOCD is the chief regulator of VSMC differentiation, although other TCFs have been identified to interact with SRF, including co-activators and co-repressors, such as KLF4 and ETS-like-1 protein (ELK-1).[69]

The pluripotency factor KLF4 is a major regulator of VSMC phenotype modulation.[11] KLF4 represses expression of contractile genes via different mechanisms,[74] including: (i) binding to the G/C repressor element of contractile gene promoters, (ii) recruitment of histone deacetylases (HDAC2/5), causing transcriptional repression of contractile genes, (iii) binding to SRF, thereby antagonizing SRF binding to CArG-boxes, and (iv) complexing with phosphorylated ELK1 and SRF, thereby

antagonizing MYOCD binding to SRF. KLF4 induces VSMC transdifferentiation into macrophage-like and MSC-like phenotypes and promotes atherosclerosis.[11] Moreover, KLF4 contributes to VSMC switching into LGALS3^{+} chondrocyte-like cells,[37] and osteogenic cells by binding to and cooperating with RUNX2.[75] The relative abundance of Runx2 and Sox9 regulates differentiation into osteogenic or chondrogenic lineages, respectively.[57] Octamer binding transcription factor (OCT4) is another pluripotency factor that regulates phenotypic switching, but has opposite effects to KLF4 (*Figure 3*).[76] Indeed, plaques of VSMC Klf4 vs. Oct4 knockout mice have virtually opposite gene expression patterns and their putative target genes play an important role in regulating VSMC phenotypic changes.[37] In contrast to Klf4, Oct4 reduces atherogenesis and promotes fibrous cap VSMC investment, potentially via increased migration.[76] Oct4 activation in VSMCs is associated with hydroxymethylation of the promoter and transcriptional regulation via hypoxia-inducible factor 1 alpha and KLF4.[76]

Other TCFs regulate VSMC differentiation through MYOCD (*Figures 1 and 3*). For example, TCF21 binds to the Myocd promoter, reducing its expression and interfering with the formation of the MYOCD-SRF complex.[77] The nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) p65 subunit binds to the Myocd promoter, decreasing its expression; repression of Myocd by NFκB acts in concert with KLF4,[78] while forkhead box O3a (FOXO3a) binds to the Myocd promoter and negatively regulates Myocd and contractile marker gene expression.[79]

4.2.2 MicroRNAs

miRNA143 and miRNA145 are the most well-documented microRNAs regulating the contractile phenotype of VSMCs (*Figures 1 and 3*).[80] miRNA143/145 block VSMC de-differentiation and proliferation by inhibiting Klf4 and Elk1 through binding with their mRNA 3′ UTR region.[81] miRNA143/145 expression is positively controlled by SRF and MYOCD, but reduced in injured or atherosclerotic aortas.[81] Moreover, cholesterol loading promotes VSMC transdifferentiation into macrophage-like cells via the miRNA143/145-MYOCD axis.[52] Hence, down-regulation of miRNA143/145 may promote atherogenesis through VSMC de-differentiation, while the MYOCD-induced miRNA1 can target Klf4 and promote VSMC differentiation.[82]

MicroRNAs implicated in VSMC phenotypic switching (*Figure 1*) also include miRNA221 and miRNA222.[83] miRNA221 stimulates proliferation by down-regulating the cell-cycle inhibitor p27^{Kip1}, while miRNA221 targets the proto-oncogene c-kit, which reduces MYOCD expression followed by reduced contractile gene expression.[84] miRNA221/222 also promote VSMC switching into osteochondrogenic like-cells and calcification.[85] miRNA21 and miRNA124 inhibit VSMC differentiation by down-regulating specificity protein 1;[86,87] the latter promotes a contractile VSMC phenotype by binding to the G/C repressor element in the promoter of contractile genes.

In addition to microRNAs, some long non-coding RNAs (LncRNAs) regulate VSMC proliferation and phenotype. LncRNAs regulate gene expression via different mechanisms, including through 'sponging' miRNAs. For example, the lncRNA SMILR promotes VSMC proliferation through direct regulation of mitotic processes and its expression is increased in unstable vs. stable human atherosclerotic plaques.[88] The lncRNA MALAT1 stimulates VSMC proliferation and migration[89] and promotes aortic stiffness.[90]

4.2.3 Epigenetic

Epigenetic modifications of amino acid residues on histones (e.g. acetylation and methylation) regulate contractile gene expression during VSMC phenotypic switching by altering chromatin structure and changing accessibility for TCFs[70] (*Figure 3*). For example, Acta2 and Myh11 are activated through histone acetylation during VSMC differentiation allowing SRF binding.[91] The transcriptional activity of MYOCD is enhanced by histone acetylation via p300 histone acetyltransferase (HAT) or inhibited by class II HDACs.[92] In contrast, KLF4-mediated recruitment of HDAC2/5 to the G/C repressor element represses contractile gene expression.[93] Collectively, increased histone acetylation of CarG box chromatin facilitates contractile gene expression by improving chromatin accessibility for MYOCD/SRF complexes through stearic hindering. HATs and HDACs also regulate many other VSMC processes including proliferation, migration, and matrix deposition that may influence VSMC phenotype and behaviour in atherosclerosis.[94]

MYOCD/SRF binding to CArG box chromatin is also associated with histone methylation. The histone modification H3K4me2 is enriched at the promoter of contractile genes during VSMC differentiation and is associated with increased tethering of MYOCD.[95] Interestingly, H3K4me2 of the Myh11 locus is restricted to VSMCs (*Figure 1*) in humans and mice, and persists in phenotypically modulated VSMCs that have lost their contractile markers, permitting its use to identify VSMCs in human atherosclerosis.[12] The retention of an epigenetic signature in phenotypically modulated VSMCs as a reminder remnant of their contractile state might explain the reversible nature of phenotype switching.[43]

4.2.4 Environmental stimuli

The platelet-derived growth factor (PDGF-BB) is up-regulated upon injury and promotes VSMC proliferation and de-differentiation via multiple mechanisms[69] (*Figures 1 and 3*). Through the PDGFRβ receptor, PDGF-BB stimulates ELK1 phosphorylation, which competes with MYOCD for the same docking site on SRF, suppressing contractile gene expression.[96] In addition, the effects of PDGF-BB on phenotypic switching are partially mediated via KLF4[97] and miRNA221.[84] The Wnt/β-catenin signalling pathway promotes VSMC proliferation and intimal thickening[98] and promotes VSMCs osteogenic transdifferentiation through activation of the Runx2 promoter.[99]

TGFβ is one of few growth factors that promotes VSMC differentiation (*Figures 1 and 3*). Contractile gene expression is regulated via the TCFs SMAD2 or SMAD3 that either bind to SMAD binding sites within contractile gene promoters or interact with SRF in a CArG-dependent manner.[100] Moreover, TGFβ blocks VSMC phenotypic switching by down-regulating Klf4 through miRNA143/145.[101] The latter are transcriptionally activated by TGFβ through MYOCD and CArG box binding. TGFβ is a multifunctional growth factor that regulates VSMC proliferation and hypertrophy, and has both matrix synthesising (e.g. stimulates production of collagens) and proteolytic (e.g. activates matrix metalloproteinases (MMPs)) properties, and can thus have secondary effects on VSMC phenotype.[69]

Recently, integrin β3 has been shown to regulate VSMC phenotype and clonality through cell-autonomous and paracrine effects.[19] Integrin β3 is produced by VSMCs and macrophages, and inhibits VSMC transdifferentiation into macrophage-like cells and limits VSMC proliferation and migration (*Figure 1*). In atherosclerosis, integrin β3 prevents clonal expansion of VSMC-derived cells in the fibrous cap and lesion, suggesting that macrophages may regulate the recruitment of medial VSMCs into the developing lesion via secretion of integrin β3.[19]

5. Role of VSMCs in atherosclerosis

By adopting different phenotypes, it is clear that VSMCs might have beneficial or maladaptive roles in atherogenesis (*Figure 2*) and plaque stability (*Figure 3*). Phenotypically modified VSMCs can also secrete a wide range of cytokines that can influence neighbouring cells in a paracrine manner. Although VSMCs can revert to their contractile phenotype under certain conditions,[38,45] there is no evidence to date that intimal VSMCs can migrate back to the media. Hence, the final content of VSMCs within the lesion is determined by the balance between VSMC immigration, proliferation, death, and senescence. We will therefore review the role of these processes in atherosclerosis, and particularly insights gained from recent lineage-tracing and scRNA-seq studies.

5.1 VSMC growth and motility

5.1.1 VSMC migration

VSMC migration from media to intima is an established part of most theories of atherogenesis, but there are multiple unanswered questions about its precise role and regulation. For example: (i) what is the contribution of migration to proliferation in human (or indeed mouse) atherosclerosis, (ii) does migration depend upon contractile-synthetic dedifferentiation, and (iii) does migration of VSMCs occur before proliferation, or can both processes occur simultaneously. In general, VSMCs have been described to migrate into the intima followed by proliferation and formation of the fibrous cap;[42,44,102] migration of medial VSMCs may be preceded by proliferation[103] or both dividing and non-dividing VSMCs can migrate and contribute to lesion formation.[23] However, lineage-tracing studies showing VSMC monochromatic clonal patches in plaques instead of the medial mosaic suggests that VSMC migration occurs independent of proliferation, and is not a major contributor to lesion formation.[13,19,27] Similarly, the observation that VSMC-derived neointimal patches are connected to medial patches expressing the same reporter colour suggests that VSMC proliferation already occurs in the media and thus precedes migration.[27] Although suggestive, these theories are based on indirect methods of exclusion of proliferative clonally expanded VSMCs; we therefore need new methods to directly monitor VSMC migration to reveal its true contribution to atherosclerosis.

5.1.2 VSMC proliferation

Historically, VSMCs were thought to accumulate in atherosclerotic lesions by ongoing proliferation in response to injury or inflammatory cues. However, VSMCs in or derived from advanced atherosclerotic plaques exhibit low proliferative indices,[104,105] and recent lineage-tracing studies might explain this proliferation paradox. For example, lineage-tracing indicates that VSMC proliferation initiates in the media and VSMC progeny then migrate into the intima, where they continue to divide in an oligoclonal fashion (*Figure 2*).[27] These few VSMC clones are highly proliferative and form the fibrous cap and invade the plaque core,[19] possibly reflecting migration of ACTA2^{+} fibrous cap cells. Multiple rounds of cell division to form large oligoclonal plaques potentially explain the cellular senescence (and therefore low proliferative indices) observed in advanced atherosclerotic plaques.

5.1.3 VSMC senescence

Cellular senescence is defined as a state of irreversible growth arrest and categorized as replicative senescence, due to replicative exhaustion of cell lifespan, or stress-induced premature senescence induced by many external stimuli, such as DNA-damaging agents or oxidative stress.[106] Both types of senescence activate a DNA damage response to initiate growth arrest and a senescent secretory phenotype (SASP) that creates a pro-inflammatory environment (see Section 5.2.3).[106] Human plaque VSMCs show reduced proliferation, early senescence, and up-regulation of cell-cycle arrest markers including p16^{INK4a} and p21, associated with hypophosphorylation of the tumour suppressor retinoblastoma protein.[107] Moreover, human plaque VSMCs have much shorter telomeres than medial VSMCs.[107] VSMC senescence promotes atherosclerosis and features of plaque instability in mice when genes promoting senescence are expressed from the Tagln promoter,[108,109] potentially driven via an IL1α-dependent SASP,[110] but it is unclear whether different VSMC phenotypes show similar levels of cell senescence and how they are regulated. Nevertheless, there is strong evidence linking VSMC senescence and aging with medial calcification.[57] For example, replicative senescence[111] or IL1β-induced senescence[112] of VSMCs enhances calcification through initiating the transition to an osteoblastic phenotype *in vitro*.

5.2 VSMC secretory and paracrine function

VSMCs secrete a variety of bioactive molecules, including matrix proteins and pro-inflammatory mediators. Some of these molecules are packed into vesicles and shed from the cell surface to transfer signals between cells. The secretome of phenotypically switched VSMCs may contain crucial information from the donor cell and even dictate the fate of the recipient cell. Unravelling the VSMC secretome may lead to the identification of specific molecules that can be used as future CVD biomarkers.

5.2.1 ECM proteins

VSMCs produce ECM as a major structural component of the vessel wall.[113] The mechano-elastic properties of arteries are largely maintained by three ECM types: elastic fibres, fibrillar collagen, and large aggregating proteoglycans, but the latter can also promote atherosclerosis by retaining apolipoprotein B-lipoproteins.[113] The interaction between VSMCs and ECM is a dynamic, bi-directional process, and ECM content is determined by the balance between production and degradation. In early plaque development, MMPs are essential for VSMC migration by degrading the cage of connective tissue surrounding VSMCs.[114] However, both the type and amount of ECM synthesised varies between VSMC phenotypes, and cultured synthetic VSMCs, macrophage-like VSMCs and VSMC-derived foam cells secrete proteolytic MMPs.

Recent advances in proteomics have enabled significant progress in our understanding of the role of ECM in atherosclerosis. For example, a 4-protein signature comprising MMP9, S100A8/S100A9, cathepsin D, and galectin-3-binding protein can differentiate symptomatic vs. asymptomatic carotid plaques, and could be used as biomarkers for risk stratification of CVD patients.[115] Similarly, a set of fibrous cap-related proteins including vitronectin, TGFβ-induced protein, complement factor 7, and apolipoprotein B have been identified in the plasma of CAD patients.[116] Mouse atherosclerotic aortas show an osteoclast-specific signature among known collagenases and MMPs,[117] and a few novel ECM factors, such as matrilin-2 and peroxidasin, although their exact role in atherosclerosis and relation to VSMCs is unclear. Proteomic profiling of secreted proteins from an atherosclerotic swine model revealed differential expression of many proteins related to VSMC activation or migration.[118] Chitinase-3-like-protein 1 in particular was up-regulated in plaque VSMCs in relation to disease stage, and in plasma of fat-fed animals vs. controls. The advances in proteomics and emerging discoveries

regarding the role of ECM in the atherosclerosis pave the way for the development of novel clinical disease biomarkers.

5.2.2 Pro-inflammatory mediators

Synthetic VSMCs play a major role in forming and maintaining the fibrous cap by secreting ECM, yet their overall role in plaque stability could be detrimental. Synthetic VSMCs secrete a wide variety of pro-inflammatory molecules and matrix-degrading enzymes that can cause cell death of neighbouring cells.[42,114] Pro-inflammatory cytokines, such as IL1β, IL6, and MCP1 promote atherogenesis by stimulating monocyte recruitment and cell death[119] and synthetic VSMCs express a range of adhesion molecules and receptors (e.g. Toll-like-receptors) that promote monocyte recruitment and regulate intracellular inflammatory signalling, respectively. Moreover, synthetic VSMCs secrete extracellular vesicles (EVs) that can drive vascular calcification (see Section 5.2.4).[59] Hence, synthetic VSMCs are beneficial for fibrous cap formation, but depending on the local environment and the disease stage, may promote inflammation, calcification, cell senescence, and plaque instability.

5.2.3 SASP

Senescent cells secrete a spectrum of cytokines, chemokines, and growth factors known as SASP, that drives many age-related diseases including atherosclerosis by sustaining a state of chronic low-grade inflammation, called 'inflammaging'.[120] Clearance of senescence cells by immune cells can be impaired in disease, propagating their accumulation.[121] The SASP can self-amplify its secretory network in an autocrine interleukin-dependent manner,[122] and induce paracrine senescence mediated via TGFβ[123] or via EVs (see Section 5.2.4).[124] Through their SASP, senescent cells attract immune cells and induce proliferation, apoptosis, or alter the differentiation status of neighbouring healthy cells,[106] including phenotypic transition of neighbouring VSMCs. For example, IL6 released by senescent human VSMCs favours their transition to an osteogenic phenotype[125] but may also promote reprogramming of neighbouring cells into a stem cell-like phenotype,[126] implying that a small number of senescent cells can have marked effects on disease development.

5.2.4 VSMC-derived vesicles

VSMCs shed different types of EVs, which can be categorized based on their size: exosomes<micro-vesicles<apoptotic bodies. EVs can mediate cell–cell communications and play important roles in both physiological and pathophysiological processes.[127] For example, EV contents include bioactive molecules, such as lipids and proteins, but also DNA, mRNA, microRNA, and lncRNA.[128] In addition to transferring cargo, EVs interact and exchange crucial information with the target cell, to change their phenotype or even reprogramme the recipient cell.[128]

Synthetic VSMCs in particular secrete exosomes and may drive calcification when the EV contains phosphatidylserine (PS), Annexin A6, and low concentrations of calcification inhibitors.[129] By exposing PS on the surface, and possibly tissue factor, exosomes may also promote vascular coagulation.[129] Matrix vesicles containing remnants of apoptotic VSMCs can act as a nucleation site for calcification in plaques,[130] and osteochondrocyte-like VSMCs secrete calcifying vesicles that further propagate calcification.[60] In addition, senescent cells secrete increased EVs compared to non-senescent cells,[124,131] which promote paracrine senescence,[124] cell proliferation, inflammation, wound healing repair, and DNA damage response pathways.[124,131,132]

5.3 VSMC death

Different forms of VSMC death occur in the atherosclerotic plaque, including apoptosis, necrosis, and various forms of regulated necrosis (e.g. necroptosis and ferroptosis).[113,134] However, with the exception of calcifying VSMCs, the susceptibility of different phenotypically modulated VSMCs to cell death is not known, in large part because of difficulties in markers for both cell death and VSMCs *in vivo*.

5.3.1 Apoptosis

Apoptotic indices are low in early plaques but increase as lesions become more advanced[135] and are higher in symptomatic plaques compared to stable lesions.[136] Apoptotic debris accumulates particularly in the core region, but apoptosis markers are transient, such that apoptotic indices do not measure apoptosis rates or efficiency of apoptotic cell clearance, and can label necrotic cells.[137] Given these limitations, the effects and incidence of apoptosis of different VSMC phenotypes are mostly unknown. VSMC apoptosis driven by expression of a suicide gene from the Tagln promoter accelerates atherogenesis, and promotes plaque vulnerability, calcification, and medial degeneration,[138] suggesting that apoptosis of medial contractile VSMCs or myofibroblast-like VSMCs in the intima is detrimental. However, further delineation of the effects of apoptosis of different VSMCs requires identification of phenotype-specific TCFs for selective cell killing.

5.3.2 Necrosis

Necrosis has long been considered an accidental and uncontrolled form of cell death. However, recent studies demonstrate that necrosis is a regulated cell death mechanism, with subtypes, such as necroptosis regulated by receptor interacting protein kinases 1 and 3 and ferroptosis characterised by accumulation of lipid-peroxidation products and lethal amounts of reactive-oxygen species derived from iron metabolism.[133] Necrosis in atherosclerosis is triggered by various stimuli, such as high levels of reactive-oxygen species, highly oxidised LDL, depletion of cellular energy, increased intracellular calcium levels, and mitochondrial Ca^{2+}-overload.[134] Inefficient efferocytosis of apoptotic VSMCs also leads to secondary necrosis and IL1-driven inflammation.[139]

Plaque cell necrosis contributes to plaque vulnerability by releasing pro-inflammatory mediators (e.g. IL1α), MMPs, and thrombogenic factors (e.g. tissue factor) whereas inhibition of necrosis improves features of plaque stability in mice.[134] However, lineage-tracing studies suggest that we need to re-evaluate the role of necrosis in atherosclerosis. Plaque cell necrosis is usually quantified by measuring the necrotic core, and necrotic cells have been reported to be predominantly (foam cell) macrophages. However, the necrotic core also contains lipid material (membrane remnants, cholesterol esters, and crystals) and lineage-traced VSMC-derived cells.[13] The precise incidence and role of necrosis in different VSMC phenotypes will require studies using lineage-tracing coupled to specific markers of necrosis *in vivo*.

6. Future perspectives

6.1 Practical recommendations for the analysis of plaque VSMCs

The observation that contractile proteins are down-regulated in atherosclerotic plaques and expressed by cells of non-VSMC origin has led to the general agreement that these contractile markers cannot be used alone to definitively identify VSMCs in plaques. Similarly, VSMCs

undergoing phenotypic modulation can express markers conventionally used for macrophages, MSCs, adipocytes, and osteochondrocyte-like cells. In contrast, a more accurate assessment of VSMCs and their different phenotypes can be achieved by lineage-tracing using permanent markers of VSMC origin inherited by their progeny coupled with protein markers (e.g. ACTA2, MYH11, TAGLN, or CD68) and transcriptional signatures obtained by scRNA-seq or *in situ* transcriptomics. Although we know some of the regulatory pathways that determine transition from contractile to synthetic phenotypes, and can postulate that particular VSMC phenotypes have a positive, neutral or negative effect on atherogenesis and overall plaque stability (*Figure 3*), currently we lack experimental proof of the role of specific phenotypes from animal studies, or correlation with events in human studies. In the meantime, surrogate markers of plaque stability, such as fibrous cap thickness, inflammatory cell content within the cap, relative necrotic core size, and neovascularisation can still be valuable indicators.

6.2 VSMC-associated CAD risk

Atherosclerosis is a complex disease controlled by both genetic and environmental factors, and more than 300 genomic loci are associated with CAD risk in humans.[140] Interestingly, approx. three-fourth of these loci are not associated with typical (lipid-related) CAD risk factors[141] and may regulate their effects through VSMCs. For example, the risk locus 9p21.3 is the most strongly associated with CAD.[142] 9p21.3 risk variants overlap the 3' region of the non-coding RNA *ANRIL* and are associated with reduced expression of the genes *CDKN2A* (encodes $p16^{INK4A}$ and $p14^{ARF}$) and *CDKN2B* (encodes p15 and $p16^{INK4B}$) located downstream of the SNPs,[142] both of which regulate VSMC proliferation and senescence. Similarly, multiple SNPs at chromosome 6q23.2 are located in the *TCF21* gene.[143] Increased *TCF21* expression is associated with reduced CAD risk and VSMC phenotypic modulation, particularly the formation of myofibroblast-like cells.[36] ATACseq experiments revealed that open chromatin regions in hVSMCs in particular are enriched for CAD risk variants[144] and TCF21 binding sites.[145] Indeed, quantitative trait variants located in CAD causal loci show allele-specific TCF21 binding and regulate chromatin accessibility and looping, suggesting that CAD-associated functional variants may underlie disease risk through regulating chromatin state.[145] Many other genes involved in regulating VSMC phenotypic switching have also been identified as CAD-associated genes by combining genome-wide association studies (GWAS) with transcriptomic and epigenetic profiling, including *KLF4, PDGFRA, SIPA1, FES*, and *SMAD3* with SMAD3 and TCF21 exerting opposing roles.[146] In addition to being a GWAS CAD gene, KLF4 might also regulate VSMC phenotype by regulating several previously reported CAD-associated gene loci.[37] ECM genes, such as collagen type 4 alpha 1/2, fibronectin and integrin β5 are also found among CAD risk loci.[113] Each of these genes will require functional characterisation of the causal variants and VSMC-specific manipulation *in vivo* to determine whether and how they regulate VSMC phenotype, atherogenesis, and features of plaque stability.

6.3 Therapeutic targeting of VSMC phenotypic switching

The integration of VSMC fate mapping and single-cell transcriptomics data from mouse studies with human single-cell genomics should allow identification of new candidate targets for therapeutic intervention. Current therapies for atherosclerosis focus mostly on lipid lowering, but the impact on plaque VSMCs and phenotypic switching is indirect, very limited, or simply unknown. For example, statins reduce foam cell formation,[147] but whether VSMC-derived foam cells are targeted as efficiently as myeloid-derived foam cells is unknown. Statins promote plaque macrocalcification[148] and delay VSMC senescence,[149] and inhibition of proprotein convertase subtilisin/kexin type 9 prevents PDGF-induced VSMC switching *in vitro*,[150] but whether this occurs though direct regulation of VSMC phenotype is unclear. We believe that the crucial role of different VSMC phenotypes in lesion development and plaque stability should focus new therapeutic strategies on stimulating the accumulation of 'plaque stabilising' VSMC phenotypes, eliminating the subpopulation of harmful VSMC phenotypes (e.g. pro-inflammatory, macrophage-like VSMCs), or switching detrimental to beneficial phenotypes (*Figure 3*), as recently shown by activation of retinoic acid signalling pathways.[38] This study nicely demonstrates the strength of combining multi-genomic techniques with fate mapping tools, the remarkable overlap in VSMC phenotypes between mouse and human atherosclerosis, the reversible nature of VSMC phenotypes, and the pharmacological application to revert VSMCs into a more stable phenotype to improve plaque stability.

7. Conclusion

For many years, VSMCs have been underestimated, misidentified, and routinely branded as either promoting atherogenesis or stabilising plaques, or both. Recent advances in VSMC lineage-tracing and the new transcriptomics means we need to re-assess this situation. However, there are still many unanswered questions. For example, how are the processes of VSMC proliferation, migration, and phenotypic switching connected, and do they differ in the media and intima; how is phenotypic switching determined spatially in the atherosclerotic plaque; what are the functions of the different phenotypes; what are the equivalent processes in human atherosclerosis, and how many and how do CAD loci regulate VSMC switching? In particular, if (as suspected) phenotypic switching of VSMCs actively contributes to plaque progression and in many cases leads to plaque instability, what are the therapeutic options to convert VSMCs into an athero-protective phenotype? The next step is to integrate VSMC-specific fate mapping and single-cell transcriptomics with human genetics to reveal novel regulatory pathways and identify candidate targets to therapeutically modulate VSMC phenotypic switching. Therapeutic approaches should be tailored to target solely the subpopulation of mischievous VSMCs by either reversing their phenotype or reprogramming the cell to redirect them into athero-protective VSMCs.

Conflict of interest: none declared.

Funding

This work was supported by British Heart Foundation Grants PG/16/11/32021, RG71070, RG84554, the National Institute of Health Research Cambridge Biomedical Research Centre, and the BHF Cambridge Centre for Research Excellence.

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